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Effects of Lipid Extraction on $\delta^{13}C$ and $\delta^{15}N$ Values in Seabird Muscle, Liver and Feathers

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Abstract.- We determined whether lipid extraction of seabird muscle, liver and feathers is required for $\delta^{13}C$ and $\delta^{15}N$ analysis, based on a study of Barau's Petrels (*Pterodroma baraui*) and White-tailed Tropicbirds (*Phaethon lepturus*). Samples were analyzed for stable isotopes before and after lipid removal. Lipid-free muscle and liver were significantly enriched in ^{13}C compared to those containing lipids (0.52 ‰ and 0.61 ‰ mean differences in $\delta^{13}C$ respectively) implying that lipids should be extracted from these tissues to avoid effects of uncontrolled differential lipid content. However lipid extraction also slightly increased muscle $\delta^{15}N$ values in tropicbirds. Researchers should thus run samples separately for $\delta^{13}C$ and $\delta^{15}N$ analysis, extracting lipids only in aliquots in which $\delta^{13}C$ is measured, or apply arithmetic $\delta^{13}C$ normalization methods on non lipid-extracted samples. Predictive models developed from our data, inferring $\delta^{13}C$ values of lipid-extracted material from $\delta^{13}C$ values and C:N ratios of non lipid-extracted material are presented and compared to five normalization methods taken from literature. Our models showed the best prediction efficiencies followed by that developed by McConnaughey and McRoy (1979). In feathers, no difference in stable isotopic measurements were noted between aliquots treated with sodium hydroxide or 2:1 chloroform:methanol. Both protocols seem suitable for preparing feather samples for isotopic analysis.

Key words.- Carbon-13, Lipid extraction, Marine birds, Nitrogen-15, Stable isotopes.

Running head: Lipid extraction for isotope analysis

In waterbird ecology stable carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotopic analysis is increasingly being used as a tool to delineate dietary patterns (Nisbet *et al.* 2002; Dahl *et al.* 2003; Cherel *et al.* 2005b; Quillfeldt *et al.* 2005), trophic relationships (Thompson *et al.* 1999, Forero and Hobson 2003, Dehna *et al.* 2006) or migratory routes (Cherel *et al.* 2000, Rubenstein and Hobson 2004). All of these applications take advantage of natural variations in stable isotope ratios resulting from chemical or biological processes and of the predictability of the stable isotopic compositions of consumer tissues in relation to that of their diet (DeNiro and Epstein 1978, Kelly 2000, Vander Zanden and Rasmussen 2001). Prior to isotopic analysis in birds, most authors extract lipids contained in tissues (Kelly 2000) as they can induce a bias in $\delta^{13}C$ readings because i) lipids are typically more depleted in ^{13}C relative to other tissue components (DeNiro and Epstein 1977) resulting in differences in $\delta^{13}C$ of around 6 – 8 ‰ (DeNiro and Epstein 1977; McConnaughey and McRoy 1979) and ii) considerable heterogeneity in lipid content exists among animals and among tissues within an organism (McConnaughey and McRoy 1979; Sweeting *et al.* 2006; Post *et al.* 2007). However, the diversity of lipid extraction procedures employed in isotopic studies is of concern as the efficiencies of each procedure may differ. Moreover, lipid extractions using the Bligh and Dyer (Bligh and Dyer 1959), the Folch (Folch *et al.* 2003) or the Soxhlet (Soxhlet 1879) methods have been shown to remove some nitrogenous compounds which, in some cases, results in changes in $\delta^{15}N$ values (Pinnegar and Polunin 1999; Cherel *et al.* 2005a; Sweeting *et al.* 2006). Nevertheless, in certain tissues with low lipid content, isotopic signatures do not differ significantly between lipid-free and bulk aliquots. It has, for example, been established that there is no need to extract lipids before isotopic analysis in avian whole blood (Bearhop *et al.* 2000; Cherel *et al.* 2005a). Here, we

determined i) whether the removal of lipids from seabird muscle, liver and feathers is a needed prerequisite for $\delta^{13}C$ analysis and ii) whether lipid removal has an effect on $\delta^{15}N$ values based on the study of two tropical species: Barau's Petrel (*Pterodroma baraui*) and White-tailed Tropicbird (*Phaethon lepturus*). To this effect, values of $\delta^{13}C$ and $\delta^{15}N$ resulting from the analysis of bulk sample material were compared to values obtained from the analysis of an aliquot from which lipids were extracted using cyclohexane for muscle and liver, and using a modified Folch rinse for feathers. In the case where the two previous assertions are true, potential solutions are considered.

METHODS

Bird Sampling and Tissue Preparation

Barau's Petrels and White-tailed Tropicbirds were sampled in Reunion Island, a tropical island situated 700 *km* east of Madagascar (21°7'*S* ; 55°33'*E*). All birds died accidentally, mainly by collision with urban lights and by poaching. Since these birds were found before or shortly after their death, we are confident that the conditions in which the birds died had no effect on carbon and nitrogen in their tissues.

Two age classes were determined (juvenile and adult) using characteristic features of the beak and the feathers (Barré *et al.* 1996, authors unpubl. data). Liver, kidney and pectoral muscles were removed, weighed and stored at -20°C. Breast feathers were also sampled on each bird. Emaciation was estimated by evaluating the muscular condition (MC) which was given an index from one to three in relation to the shape of the pectoral muscles: one, well developed pectoral muscles; two,

slight pectoral muscle atrophy; three, severe pectoral muscle atrophy (Bolton *et al.* 1991). Body condition (BC) was also evaluated using a condition index proposed by Wenzel and Adelung (1996). This condition index is the ratio of liver to kidney masses and is significantly negatively correlated to the degree of emaciation of the bird (Debacker *et al.* 2001). The healthiest birds were selected for this study: MC equal to one or two, and mean BC equal to 324 ± 41 for Barau's Petrels and 255 ± 68 for White-tailed Tropicbirds. Muscle samples originated from 20 adult Barau's Petrels and 24 White-tailed Tropicbirds, and liver samples from 20 Barau's Petrels and 20 White-tailed Tropicbirds. Breast feathers came from ten Barau's Petrels.

To prepare for isotopic determination, livers and muscles were frozen at -80°C, freeze dried and ground to fine powder. Feathers were washed vigorously in triple baths of 0.25 N sodium hydroxide solution alternated with triple baths of deionized water, a method commonly used to remove external contamination as it may interfere with isotopic measurement results (Walsh 1990; Thompson *et al.* 1998; Schreiber and Burger 2001). Feathers were then dried in an oven for 24 h at 50°C.

Lipid Extraction

Each sample was divided into two aliquots. For the first aliquots, the preparation ended at the protocol step described above. The second aliquots were submitted to lipid extraction processes commonly employed in bird studies (e.g. Hobson *et al.* 2002; Sagerup *et al.* 2002; Cherel *et al.* 2005a).

Lipid extraction in muscle and liver was performed using 20 ml of cyclohexane on powder

aliquots of about 1 g. An ultra Turax was used to homogenize the mixture (2 x 15 sec). The sample was then centrifuged for 2 min at 804 *g*. The supernatant containing the lipids was then disposed off, whereas the pellet was dried on an aluminum plate for 12 h at 60°C. All utensils were washed with detergent, then with ethanol, rinsed with deionized (Milli-Q quality) water and dried in an oven at 60°C before use.

A modified Folch method was applied to the feathers. They were soaked in a 2:1 chloroform:methanol rinse (still for 30 min, followed by five min sonication). Feathers were then dried in an oven and cut into small fragments.

Isotopic Analysis

Stable carbon and nitrogen isotope assays were carried out on 1 ± 0.02 mg subsamples of powder loaded into tin cups. Continuous-flow isotope-ratio mass spectrometry (CF-IRMS) analyses were conducted using a Europa Scientific ANCA-NT 20-20 Stable Isotope Analyzer with ANCA-NT Solid/Liquid Preparation Module (Europa Scientific Ltd., Dundee, UK). Every ten samples were separated by two laboratory standards (leucine) which were calibrated against "Europa flour" (potato flour) and IAEA standards N1 and N2 (Scrimgeour and Robinson 2003). Sample analysis also yielded carbon and nitrogen percentages from which carbon-to-nitrogen (C:N) ratios (by weight) were derived. Experimental precision (based on the standard deviation of replicate measurements of the internal standards) for $\delta^{13}C$, $\delta^{15}N$, %C and %N was 0.07 ‰, 0.12 ‰, 1.61 % and 0.29 % respectively.

Stable isotope results are expressed in delta notation (δ), defined as the part per thousand (‰) deviation from a standard material:

$$\delta = [(R_{sample} / R_{standard}) - 1] \times 1000$$

where R_{sample} and $R_{standard}$ are the fractions of heavy to light isotopes in the sample and standard, respectively. The international standards are, the Pee Dee Belemnite (PDB) marine fossil limestone formation from South Carolina for $\delta^{13}C$, and atmospheric nitrogen for $\delta^{15}N$.

Statistical analysis

Statistical analyses were performed using the GNU R statistical system (R Development Core Team 2005). Data were first checked for normality and homogeneity of the variances by means of Shapiro-Wilk and Bartlett tests respectively. Wilcoxon and *t*-tests for paired samples were used accordingly to test the significance of differences of the isotopic deviations and the C:N ratios between aliquots. Isotopic deviation results are reported as means and standard deviations (SD).

RESULTS

Muscle and Liver

Results presented in Table 1 show that lipid extraction significantly affected $\delta^{13}C$ values in muscle and liver of both species, lipid-free aliquots being enriched in ^{13}C by, on average, 0.52 ± 0.56 ‰ in muscle and 0.61 ± 0.98 ‰ in liver in comparison with aliquots containing lipids. The largest

mean difference between bulk ($\delta^{13}C_{bulk}$) and lipid-extracted ($\delta^{13}C_{ext}$) $\delta^{13}C$ values (3.22 ‰) was seen in juvenile Barau's Petrel liver where lipid content seemed to be the highest (C:N ratio of 5.81). The only case where $\delta^{13}C_{ext}$ values were not significantly different from $\delta^{13}C_{bulk}$ values was noted when the tests were restrictively applied to adult Barau's Petrel liver. Of the 84 samples, nine showed very slightly negative value for the difference between δ^{13}Cext and δ^{13}Cbulk values ($\Delta\delta^{13}C$); this deviation below the theoretical value of zero for lipid-free tissue was close to the analytical precision. Furthermore $\Delta\delta^{13}C$ were positively correlated in a linear fashion with C:N ratios in bulk material from both tissues (Figure 1). After lipid extraction, C:N ratios in both species dropped close to a value of 4 in both tissues with average variation becoming lower (Table 1); the average post-extraction C:N ratio of all seabirds was 3.90 ± 0.14 in muscle and 4.06 ± 0.23 in liver.

Stable nitrogen isotope values were affected by lipid extraction only in White-tailed Tropicbird muscle in which lipid-extracted $\delta^{15}N$ ($\delta^{15}N_{ext}$) values were, on average, 0.20 ‰ higher than bulk $\delta^{15}N$ ($\delta^{15}N_{bulk}$) values (Table 1).

Feathers

Feather $\delta^{13}C$ and $\delta^{15}N$ values did not differ significantly between the samples washed in sodium hydroxide baths and the corresponding aliquots having undergone sodium hydroxide and 2:1 chloroform:methanol baths (Table 1), although C:N ratio decreased slightly but significantly ($p = 0.006$). Intra-specific variability in C:N ratios was very small, even for bulk samples (Table 1).

DISCUSSION

Muscle and Liver

The enrichment of ^{13}C with lipid extraction observed in Barau's Petrel and in White-tailed Tropicbird tissues were lower than the 1.3 ‰ value reported by Kelly (2000) for the muscle of carnivorous seabirds and consistent with the 0.61 to 0.67 ‰ increase noted in fish muscle (Pinnegar and Polunin 1999; Sweeting *et al.* 2006). These results suggest that lipids present in Barau's Petrel and White-tailed Tropicbird muscular and hepatic tissues alter their stable carbon isotope signatures. This experiment adds to the growing body of evidence supporting lipid extraction as a prerequisite to $\delta^{13}C$ determination in lipid-rich avian tissues, namely muscle and liver, as in several other organisms (Pinnegar and Polunin 1999; Kelly 2000; Sweeting *et al.* 2006; Bodin *et al.* 2007).

Lipid extraction affected $\delta^{15}N$ only in White-tailed Tropicbird muscle. Some lipids, for example glycolipids or lipoproteins, contain nitrogenous compounds. It is possible that such nitrogenous compounds or protein material were lost during the lipid extraction process, explaining the change in the muscular $\delta^{15}N$ signatures of White-tailed Tropicbirds. This average increase of 0.20 ‰ of $\delta^{15}N$ values with lipid extraction is very close to that observed by Post *et al.* (2007) when working on a variety of animals (0.25 ± 0.18 ‰). The fact that an effect of lipid-extraction was observed only in White-tailed Tropicbird muscle might be linked to differences in physiological makeup between tissues and species.

In conclusion, lipid extraction is necessary for $\delta^{13}C$ determination in seabird muscle and liver but chemical extraction may, in some cases, alter $\delta^{15}N$ values. Theoretically, such samples should be

run in separate batches of treated (for $\delta^{13}C$ determination) and untreated (for $\delta^{15}N$ determination) aliquots. Separated analysis are however time consuming and costly. An alternative to dual analysis and lipid extraction is the use of arithmetic correction techniques for predicting $\delta^{13}C_{ext}$ values. These include lipid normalization models that predict $\delta^{13}C_{ext}$ values ($\delta^{13}C_{predicted}$) from sample $\delta^{13}C_{bulk}$ values and C:N ratios (McConnaughey and McRoy 1979; Alexander *et al.* 1996; Kiljunen *et al.* 2006; Bodin *et al.* 2007; Post *et al.* 2007). These equations present the advantage not to require information neither on lipid content, lipid $\delta^{13}C$ signatures nor on C:N ratios in protein. The three most commonly used lipid normalization models are of the following form:

$$\delta^{13}C_{predicted} = \delta^{13}C_{bulk} + D\ ((I + 3.9) / (1 + 287 / L)) \quad \text{Eqn 1}$$

$$\delta^{13}C_{predicted} = \delta^{13}C_{bulk} + D\ (L / 100) \quad \text{Eqn 2}$$

$$\delta^{13}C_{predicted} = a\ (C:N) + b + \delta^{13}C_{bulk} \quad \text{Eqn 3}$$

In these equations, L, a function of C:N, is an approximation of the proportional lipid content of the bulk sample established by McConnaughey and McRoy (1979): $L = 93 / (0.246 \times (C:N) - 0.775)^{-1}$. I, a and b are parameters estimated from data. D is the difference in $\delta^{13}C$ between protein and lipid; it is sometimes estimated from data and sometimes fixed to 6 ‰, a value derived from published data (McConnaughey and McRoy 1979; Alexander *et al.* 1996). Note that Equation 1 can be equivalently rewritten as:

$$\delta^{13}C_{predicted} = \delta^{13}C_{bulk} + D\ I + D\ f(C:N) \quad \text{Eqn 4}$$

where $f(C:N) = 3.9 / (1 + (287 / 93)\ (1 + (1 / 0.246\ (C:N) - 0.775)))$.

Here, in order to obtain $\delta^{13}C$ normalization models for seabird muscle and liver tissues, we fitted, by least squares, Equations 3 and 4 to the observed data (Figure 1), which led to new estimations of a and b, and D and I. Equation 4 was also fitted to the observed data by estimating only I and by assigning D a value of 6. Additionally, a modified version of Equation 3, containing a third parameter (c), was fitted to the observed data:

$$\delta^{13}C_{predicted} = a\,(C:N) + b + c\,\delta^{13}C_{bulk} \qquad \text{Eqn 5}$$

To ensure better clarity in the following discussion, models given in Equations 1 and 4 will be referred to as models of Type I, and models given in Equations 3 and 5 to models of Type II and III respectively. The values of the parameters estimated in different studies, including this one, are presented in Table 2.

In order to compare the predictive quality of the different models for the considered seabird data, we calculated the root mean squared error (rmse) between the observed and the predicted values of $\delta^{13}C$. For the models estimated from the seabird data themselves, to avoid biased results, the rmses were computed using leave-one-out cross validation: for each of the n observations (n = 44 and n = 40 for muscle and liver respectively), the $\delta^{13}C_{predicted}$ value was computed using the model estimated from the n - 1 remaining observations; the square root of the mean of the n squared errors between the predicted and the observed values was then calculated. From the rmses presented in Table 2, it appears that the leave-one-out models estimated from the observed data led, in all cases, to the lowest rmses which could have been expected. They are followed by the models proposed by McConnaughey and McRoy (1979), Bodin *et al.* (2007), Post *et al. (*2007), Alexander *et al.* (1996)

and Kiljunen *et al.* (2006) in decreasing order of fitness. The significance of the differences between the $\delta^{13}C_{predicted}$ values by these models was tested using t-tests for paired samples. The results, assuming a 5 % significance level, are presented in columns M and L of Table 2. Furthermore, the 95 % confidence intervals, and the 95 % and 99 % confidence ellipses (Faraway 2005; Murdoch *et al.* 2007) for the estimated parameters of models of Type I and II are presented in Table 2 (column 6), and in Figures 2a and 2b respectively. When confronted to the confidence ellipses, the parameters estimated by the other authors for models of Type I and II are outside the ellipses indicating significant differences between our fitted models for muscle and liver and the existing models. This was confirmed by the paired t-test results which showed that significant differences existed among $\delta^{13}C_{predicted}$ values predicted by the models of other authors in all but one case (between the models developed by Post *et al. (*2007) and Alexander *et al.* (1996) when applied to the liver data set) and that the absence of significant differences among $\delta^{13}C_{predicted}$ values for our models should not be rejected (Table 2). This suggest that, for all types of models, the parameters of the relation between $\delta^{13}C_{ext,}$ and C:N and $\delta^{13}C_{bulk,}$ strongly vary with the biological material. In Figure 3, the $\delta^{13}C_{predicted}$ values calculated using the best models (in terms of rmse) fitted from our data for muscle (Equation 3 with a = 0.828 and b = -3.147) and liver (Equation 5 with a = 0.350, b = -4.162 and c= 0.838) are plotted against the observed $\delta^{13}C_{ext}$ values and compared to $\delta^{13}C_{predicted}$ values calculated using the five other models obtained from literature. A part from the Bodin *et al.* (2007) model applied to liver, the models seem to overestimate the $\delta^{13}C_{predicted}$ values in seabird tissues. Globally, of the five models, none predicts $\delta^{13}C_{ext}$ values from $\delta^{13}C_{bulk}$ and C:N values stemming from seabird muscle or liver tissues in a very satisfactory way.

The arithmetic lipid normalization models established in this study have been estimated from seabirds originating from a single location, belonging to only two species and characterized by a fairly narrow and low range of C:N ratios. Type I models nonlinearly relate $\Delta\delta^{13}C$ to C:N because of the expectation that $\Delta\delta^{13}C$ will converge to D at high C:N ratios (McConnaughey and McRoy 1979; Alexander *et al.* 1996). If this is so, our samples might be sitting within the region where $\Delta\delta^{13}C$ and C:N are linearly related (Figure 1). Hence, as the differences in the predictive qualities of the four models estimated here were not significant, we suggest that the model of Type I (where D = 6 and I = - 0.180 and - 0.220 in muscle and liver respectively) be used in priority by researchers wanting to apply these seabird-adapted models to their samples. However, because the nature of the biological material and the range of C:N ratios seem to influence lipid normalization models, the models proposed here for seabird muscle and liver should be validated using an independent data set from other species of seabirds from various locations and habitats, and extended to a larger range of C:N ratios before being considered fit for general use in seabird studies. More generally, if researchers want to fit one of the three types of models to their data, it is recommended that they choose Type I models since their functional form seems to be the most appropriate for large C:N ranges, and they do not appear to perform significantly worse when the C:N range is small and the relationship between $\Delta\delta^{13}C$ and C:N appears as linear.

Feathers

Two hypotheses can be made to explain the absence of difference in stable isotopic signatures

between samples washed in sodium hydroxide baths and the corresponding aliquots having undergone sodium hydroxide and 2:1 chloroform:methanol baths: either i) sodium hydroxide baths are efficient in extracting lipids that cover the bird's plumage or, ii) lipids attached to the feather surface do not significantly alter isotopic signatures.

Kiljunen et al. (2006) consider that tissues deprived of lipid have a C:N ratio equal to three. C:N ratios in in Barau's Petrels' bulk feather samples were close to this value, on average equal to 3.19, indicating low lipid levels, and decreased only very slightly to 3.15 after the modified Folch treatment. Considering the first hypothesis, these results imply that sodium hydroxide baths are efficient in extracting plumage lipids but not to the extent of the modified Folch method. Ambrose (1993) showed that sodium hydroxide is, however, equally effective in removing lipids as either chloroform or diethyl ether in human tissue. Considering that sodium hydroxide baths are not efficient (second hypothesis), the above results added to the very small intra-specific variability in C:N ratios indicate that the amount of lipid spread over their feathers by these birds when preening is rather constant among individuals and small enough not affect $\delta^{13}C$ readings. In this case, our results support the conclusion made by Post *et al.* (2007) that it is not necessary to account for lipids in aquatic animal samples when lipid content is below 5 % (C:N < 3.5).

More generally, it may be concluded that either treatment can be used in view of stable carbon and nitrogen isotopic measurements in plumage. It is noteworthy that the use of 0.25 N sodium hydroxide (irritant for the eyes and the skin in case of prolongated or repeated contact) is much less hazardous to human health than the use of chloroform (noxious when inhaled, ingested or in contact with the skin; causes severe and irreversible effects) or that of methanol (toxic when

inhaled, ingested or in contact with the skin and may cause death) (Acros Organics 2004-2005) making its usage easier. Moreover, it would be of interest to compare other methods used to clean feathers (Hobson *et al.* 1993; Thompson and Furness 1995; Cherel *et al.* 2000; Becker *et al.* 2002) with the protocols tested here.

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LITERATURE CITED

Alexander, S. A., K. A. Hobson, C. L. Gratto-Trevor and A. W. Diamond. 1996. Conventional and isotopic determinations of shorebird diets at an inland stopover: The importance of invertebrates and *Potamogeton pectinatus* tubers. Canadian Journal of Zoology 74: 1057-1068.

Acros Organics. 2004-2005. Catalog of fine chemicals. Acros Organics, Noisy-Le-Grand, France.

Ambrose, S. H. 1993. Isotopic analysis in paleodiets: methodological and interpretive consideration. pages 59-130 *in* Investigation of ancient human tissue (M. K. Sanford Eds.). Gordon and Breach, Langhorne, P.A.

Barré, N., A. Barau and C. Jouanin. 1996. Oiseaux de la Réunion, Les Editions du Pacifique, Paris, France.

Bearhop, S., S. Waldreon, D. Thompson, and R. Furness. 2000. Bioamplification of mercury in Great Skua and Catharacta Skua chicks: the influence of trophic status as determined by stable isotope signatures of blood and feathers. Marine extraction and arithmetic lipid correction on stable isotope ratio of fish muscles. Rapid Communications in Mass Spectrometry 20: 595–601.

Becker, P. H., J. González-Solís, B. Behrends, and J. Croxall. 2002. Feather mercury levels in seabirds at South Georgia: influence of trophic position, sex and age. Marine Ecology Progress Series 243: 261–269.

Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. Canadian Journal of Biochemistry and Physiology 37: 911–917.

Bodin, N., F. Le Loc'h, and C. Hily. 2007. Effect of lipid removal on carbon and nitrogen stable isotope ratios in crustacean tissues. Journal of Experimental Marine Biology and Ecology 341: 168-175.

Bolton, M., P. Monaghan, and D. C. Houston. 1991. An improved technique for estimating pectoral muscle protein condition from body measurements of live gulls. Ibis 133: 264–270.

Cherel, Y., K. A. Hobson, and S. Hassani. 2005a. Isotopic discrimination between food and blood and feathers of captive penguins: Implications for dietary studies in the wild. Physiological and Biochemical Zoology 78: 106-115.

Cherel, Y., K. A. Hobson, and H. Weimerskirch. 2000. Using stable-isotope analysis of feathers to distinguish moulting and breeding origins of seabirds. Oecologia 122: 155–162.

Cherel, Y., K. A. Hobson, and H. Weimerskirch. 2005b. Using stable isotopes to study resource acquisition and allocation in procellariiform seabirds. Oecologia 145: 533–540.

Dahl, T. M., S. Falk-Petersen, G. W. Gabrielsen, J. R. Sargent, H. Hop, and R. M. Millar. 2003. Lipids and stable isotopes in common eider, Black-legged Kittiwake and Northern Fulmar: a trophic study from an Arctic fjord', Marine Ecology Progress Series 256: 257–269.

Debacker, V., T. Jauniaux, F. Coignoul, and J.-M. Bouquegneau. 2001. Heavy metal contamination and body condition of wintering guillemots (*Uria aalge*) at the Belgian coast from 1993 to 1998. Environmental Research 84: 310–317.

Dehna, L.-A., E. H. Follmanna, D. L. Thomasb, G. G. Sheffieldc, C. Rosaa, L. K. Duffya and T. M. O'Haraa. 2006. Trophic relationships in an Arctic food web and implications for trace metal transfer. Science of The Total Environment 362: 103-123.

DeNiro, M. J. and S. Epstein. 1977. Mechanism of carbon fractionation associated with lipid synthesis. Science 197: 261–263.

DeNiro M. J., and S. Epstein. 1978. Influence of diet on the distribution of carbon isotopes in animals. Geochimica et Cosmochimica Acta 42: 495-506.

Faraway, J. J. 2005. Linear Models with R, Chapman and Hall/CRC, Florida, U.S.A.

Forero, M. G., K. A. Hobson. 2003. Using stable isotopes of nitrogen and carbon to study seabird ecology: applications in the Mediterranean seabird community. Scientia Marina 67: 23-32

Folch, J., M. Lees and G. H. Sloane Stanley. 2003. A simple method for the isolation and purification of total lipids from animal tissues. Journal of Biological Chemistry 226: 497–510.

Hobson, K. A., R. T. Alisauska, and R. G. Clark. 1993. Stable-nitrogen isotope enrichment in avian tissues due to fasting and nutritional stress: implications for isotopic analyses of diet. The Condor 95: 388–394.

Hobson, K. A., A. Fisk, N. Karnovsky, M. Holst, J.-M. Gagnone, and M. Fortier. 2002. A stable isotope ($\delta^{13}C$, $\delta^{15}N$) model for the North Water food web: implications for evaluating trophodynamics and the flow of energy and contaminants. Deep-Sea Research Part II – Tropical studies in oceanography 49: 5131–5150.

Kelly, J. F. 2000. Stable isotopes of carbon and nitrogen in the study of avian and mammalian trophic ecology. Canadian Journal of Zoology 78: 1–27.

Kiljunen, M., J. Grey, T. Sinisalo, C. Harrod, H. Immonen and R. I. Jones. 2006. A revised model for lipid-normalizing $\delta^{13}C$ values from aquatic organisms, with implications for isotope mixing models. Journal of Applied Ecology 43: 1213-1222.

McConnaughey, T. and C. P. McRoy. 1979. Food-Web structure and the fractionation of Carbon isotopes in the bering sea. Marine Biology 53: 257-262.

Murdoch, D., E. D. Chow and J. M. Frias Celayeta. 2007. ellipse: Functions for drawing ellipses and ellipse-like confidence regions. R package version 0.3-5.

Nisbet, I. C. T., J. P. Montoya, J. Burger, and J. J. Hatch. 2002. Use of stable isotopes to investigate individual differences in diets and mercury exposures among common terns *Sterna hirundo* in breeding and wintering grounds. Marine Ecology Progress Series 242: 267–274.

Pinnegar, J. K. and N. V. C. Polunin. 1999. Differential fractionation of $\delta^{13}C$ and $\delta^{15}N$ among fish tissues: implications for the study of trophic interactions. Functional Ecology 13: 225.

Post, D. M., C. A. Layman, D. A. Arrington, G. Takimoto, J. Quattrochi and C. G. Montana. 2007. Getting to the fat of the matter: models, methods and assumptions for dealing with lipids in stable isotope analyses. Oecologia 152: 179-189.

Quillfeldt, P., R. A. R. McGill, and R. W. Furness. 2005. Diet and foraging areas of Southern Ocean seabirds and their prey inferred from stable isotopes: Review and case study of Wilson's storm-petrel. Marine Ecology Progress Series 295: 295-304.

R Development Core Team. 2005. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org, accessed 15 November 2006.

Rubenstein D. R., and K. A. Hobson. 2004. From birds to butterflies: animal movement patterns and stable isotopes. Trends in Ecology and Evolution 19: 256-263

Sagerup, K., E. O. Henriksen, J. U. Skaare, and G. W. Gabrielsen. 2002. Intraspecific variation in trophic feeding levels and organochlorine concentrations in Glaucous Gulls (*Larus hyperboreus*) from Bjørnøya, the Barents Sea. Ecotoxicology 11: 119–125.

Schreiber, E. A. and J. Burger. 2001. Biology of marine birds. CRC Press, USA.

Scrimgeour, C. M. and D. Robinson. 2003. Stable isotope analysis and applications. Pages 381-432 *in* Soil and Environmental Analysis: Modern Instrumental Techniques (K. A. Smith and M. S. Cresser Eds.), Marcel Dekker Inc., New York, New York, USA.

Soxhlet, F. 1879. Die Gewichtsanalytische Bestimmung des Milchfettes. Polytechnisches Journal 232: 461–465

Sweeting, C. J., N. V. C. Polunin, and S. Jennings. 2006. Effects of chemical lipid extraction and arithmetic lipid correction on stable isotope ratio of fish muscles. Rapid Communications in Mass Spectrometry 20: 595–601.

Thompson, D. R., S. Bearhop, J. R. Speakman, and R. W. Furness.1998. Feathers as a means of monitoring mercury in seabirds: insights from stable isotope analysis. Environmental Pollution 101: 193–200.

Thompson, D. R. and R. W. Furness. 1995. Stable-isotope ratios of carbon and nitrogen in feathers indicate seasonal dietary shifts in Northern Fulmars. The Auk 112: 493–498.

Thompson, D. R., K. Lilliendahl, J. Solmundsson, R. W. Furness, S. Waldron, R. A. Phillips. 1999. Trophic Relationships among Six Species of Icelandic Seabirds as Determined through Stable Isotope Analysis. The Condor 101: 898-903.

Vander Zanden M. J., and J. B. Rasmussen. 2001. Variation in 15N and 13C trophic fractionation: Implications for aquatic food web studies. Limnology and Oceanography 46: 2061-2066.

Walsh, P. M. 1990. The use of seabirds as monitors of heavy metals in the marine environment. Pages 183–205 *in* Heavy metals in the marine environment (R. Furness and P. Rainbow Eds.). CRC Press, Boca Raton, Florida.

Wenzel, C. and D. Adelung. 1996. The suitability of oiled guillemots (*Uria aalge*) as monitoring organisms for geographical comparisons of trace element contaminants. Archives of Environmental Contamination and Toxicology 31: 368–377.

TABLE 1: Stable isotope results for Barau's Petrels (*Pterodroma baraui*) and White-tailed Tropicbirds (*Phaethon lepturus*), with juveniles (J) and adults (A) considered separately and together. Number of samples (n), mean (± SD) $\delta^{13}C$ values, $\delta^{15}N$ values (‰) and C:N ratios of bulk and chemically lipid-extracted seabird muscle, liver and feathers are presented, together with the differences between lipid-extracted and bulk aliquots (± SD) followed by their significance (NS, *, **, *** indicate that the mean difference is not significant, significant at $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively).

TABLE 2: Lipid normalization models for predicting $\delta^{13}C_{ext}$ values from sample $\delta^{13}C_{bulk}$ values and C:N ratios. D and I (resp. a, b and c) are the parameters of Equation 1 and 2 (resp. 3 and 5) estimated by different authors. “rmse” stands for “root mean square error” which was calculated for each model applied to each tissue (M: muscle; L: liver). In the last two columns (M and L), different Latin or Greek letters symbolize significant differences between the $\delta^{13}C_{ext}$ values predicted by the models.

TABLE 1:

	n	Bulk			Lipid extracted				
		$\delta^{13}C$	$\delta^{15}N$	C:N	$\delta^{13}C$	$\delta^{15}N$	C:N	$\delta^{13}C_{ext} - \delta^{13}C_{bulk}$	$\delta^{15}N_{ext} - \delta^{15}N_{bulk}$
Muscle									
Pterodroma baraui A	20	−18.12 ± 0.79	13.15 ± 0.59	4.26 ± 0.54	−17.92 ± 0.60	13.12 ± 0.59	3.87 ± 0.12	0.40 ± 0.57 **	0.01 ± 0.22 NS
Phaethon lepturus A	11	−17.83 ± 0.81	11.55 ± 0.95	4.41 ± 0.59	−17.35 ± 0.43	11.82 ± 0.83	3.96 ± 0.13	0.48 ± 0.51 *	0.27 ± 0.13 ***
Phaethon lepturus J	13	−18.11 ± 0.91	11.70 ± 1.41	4.65 ± 0.71	−17.37 ± 0.57	11.75 ± 1.36	3.89 ± 0.17	0.74 ± 0.54 ***	0.14 ± 0.15 **
Phaethon lepturus A+J	24	−17.98 ± 0.86	11.63 ± 1.20	4.54 ± 0.66	−17.36 ± 0.50	11.78 ± 1.14	3.92 ± 0.16	0.62 ± 0.53 ***	0.20 ± 0.15 ***
Liver									
Pterodroma baraui A	10	−18.85 ± 1.22	15.34 ± 1.38	4.46 ± 0.49	−18.28 ± 0.98	15.25 ± 1.20	4.00 ± 0.16	0.39 ± 1.62 NS	0.08 ± 0.15 NS
Pterodroma baraui J	10	−20.51 ± 1.34	14.37 ± 0.82	5.81 ± 1.32	−19.20 ± 0.75	14.35 ± 0.75	4.20 ± 0.21	3.22 ± 6.20 ***	−0.03 ± 0.27 NS
Pterodroma baraui A+J	20	−19.64 ± 1.51	14.86 ± 1.21	5.13 ± 1.19	−18.60 ± 1.00	14.92 ± 1.14	4.08 ± 0.20	1.74 ± 4.55 *	0.03 ± 0.22 NS
Phaethon lepturus A	10	−18.06 ± 0.51	13.97 ± 0.87	4.08 ± 0.63	−17.43 ± 0.51	14.28 ± 0.96	4.09 ± 0.32	0.43 ± 0.17 ***	0.01 ± 0.12 NS
Phaethon lepturus J	10	−18.42 ± 0.58	14.44 ± 2.22	4.36 ± 0.26	−17.91 ± 0.80	14.17 ± 2.24	3.98 ± 0.14	0.44 ± 0.18 ***	0.05 ± 0.13 NS
Phaethon lepturus A+J	20	−18.25 ± 0.56	14.20 ± 1.72	4.58 ± 0.52	−17.66 ± 0.70	14.23 ± 1.68	4.04 ± 0.25	0.43 ± 0.17 ***	0.03 ± 0.12 NS
Feathers									
Pterodroma baraui A	5	−15.68 ± 1.29	13.37 ± 0.58	3.18 ± 0.02	−15.72 ± 1.05	13.30 ± 0.91	3.15 ± 0.02	−0.04 ± 0.27 NS	−0.07 ± 1.35 NS
Pterodroma baraui J	5	−16.92 ± 0.41	14.00 ± 0.36	3.21 ± 0.03	−16.61 ± 0.43	13.74 ± 0.76	3.15 ± 0.01	0.32 ± 0.04 NS	−0.25 ± 0.63 NS
Pterodroma baraui A+J	10	−16.30 ± 1.11	13.68 ± 0.57	3.19 ± 0.03	−16.16 ± 0.89	13.52 ± 0.82	3.15 ± 0.01	0.05 ± 0.19 NS	0.42 ± 0.81 NS

TABLE 2:

Model type	Eqn	Estimated equation parameters	r^2	p-value	95% CI	rsme	n	Biological material	C:N range	References	M	L
I	1	D = 6; I = -0.207	-	-	-	M: 0.277 L: 0.290	-	Range of animals and plankton (muscle, whole organisms)	-	McConnaughey and McRoy (1979)	a	α
	1	D = 7.018; I = 0.048	-	-	-	M: 1.715 L: 2.016	109	Fish dorsal muscle	2 – 63	Kiljunen *et al.* (2006)	b	β
	4	D = 5.590; I = -0.174	0.831	< 0.001	D: 4.816, 6.364 I: -0.213, -0.135	M: 0.236	44	Seabird muscle	3.7 – 6.0	Present study	c	
	4	D = 6; I = -0.180	-	-	-	M: 0.233	44	Seabird muscle	3.7 – 6.0	Present study	c	
	4	D = 4.846; I = -0.196	0.772	< 0.001	D: 3.994, 5.698 I: -0.252, -0.134	L: 0.274	40	Seabird liver	4.0 – 7.9	Present study		γ
	4	D = 6; I = -0.220	-	-	-	L: 0.286	40	Seabird liver	4.0 – 7.9	Present study		γ
II	2	D = 6	-	-	-	M: 0.793 L: 0.989	-	Shorebird muscle	-	Alexander *et al.* (1996)	d	ϵ
	3	a = 0.322; b = -1.175	-	-	-	M: 0.467 L: 0.371	46	Crustacean muscle, gonads and hepathopancreas	4.0 – 16.2	Bodin *et al.* (2007)	e	ζ
	3	a = 0.99; b = -3.32	-	-	-	M: 0.593 L: 1.010	22	Aquatic animals (muscle, whole organisms)	2.9 – 6.9	Post *et al.* (2007)	f	ϵ
	3	a = 0.828; b = -3.147	0.841	< 0.001	a: 0.718, 0.939 b: -3.642, -2.651	M: 0.232	44	Seabird muscle	3.7 – 6.0	Present study	c	
	3	a = 0.522; b = -1.926	0.810	< 0.001	a: 0.440, 0.603 b: -2.331, -1.520	L: 0.245	40	Seabird liver	4.0 – 7.9	Present study		γ
III	5	a = 0.781; b = -3.607; c= 0.963	0.883	< 0.001	a: 0.590, 0.972 b: -5.196, -2,018 c: 0.842, 1.084	M: 0.238	44	Seabird muscle	3.7 – 6.0	Present study	c	
	5	a = 0.350; b = -4.162; c= 0.838	0.957	< 0.001	a: 0.251, 0.449 b: -5.173, -3.151 c: 0.769, 0.907	L: 0.205	40	Seabird liver	4.0 – 7.9	Present study		γ

Figure captions

FIGURE 1: Relationship between C:N ratios and $\Delta\delta^{13}C$ (‰) in seabird tissues (■: *Pterodroma baraui;* ○: *Phaethon lepturus*).

FIGURE 2a: 95 % (inner ellipse) and 99 % (outer ellipse) confidence ellipses for models of Type I estimated here from seabirds muscle and liver. The parameters of the models of equivalent type fitted by other authors are plotted on these graphs (crosses).

FIGURE 2b: 95 % (inner ellipse) and 99 % (outer ellipse) confidence ellipses for models of Type II estimated here from seabirds muscle and liver. The parameters of the models of equivalent type fitted by other authors are plotted on these graphs (crosses).

FIGURE 3: Observed (chemically lipid-extracted) and predicted (from various lipid-normalization methods) $\delta^{13}C$ values (‰) from Reunion Island seabirds used for independent validation of the models. The solid line shows the 1:1 ratio for observed and predicted values.

FIGURE 1:

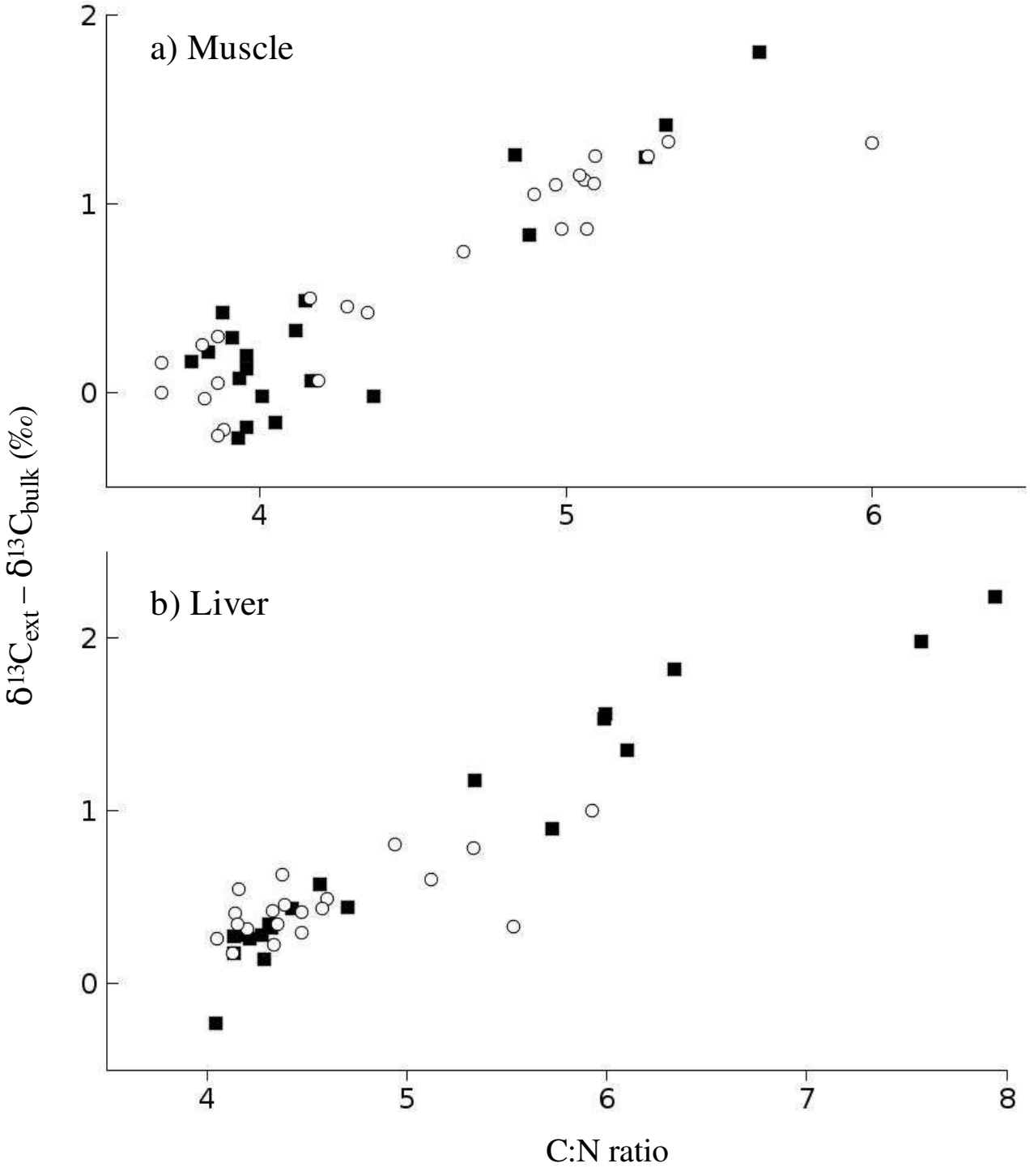

a) Muscle
b) Liver
$\delta^{13}C_{ext} - \delta^{13}C_{bulk}$ (‰)
C:N ratio
2
1
0
4
5
6
7
8

FIGURE 2a:

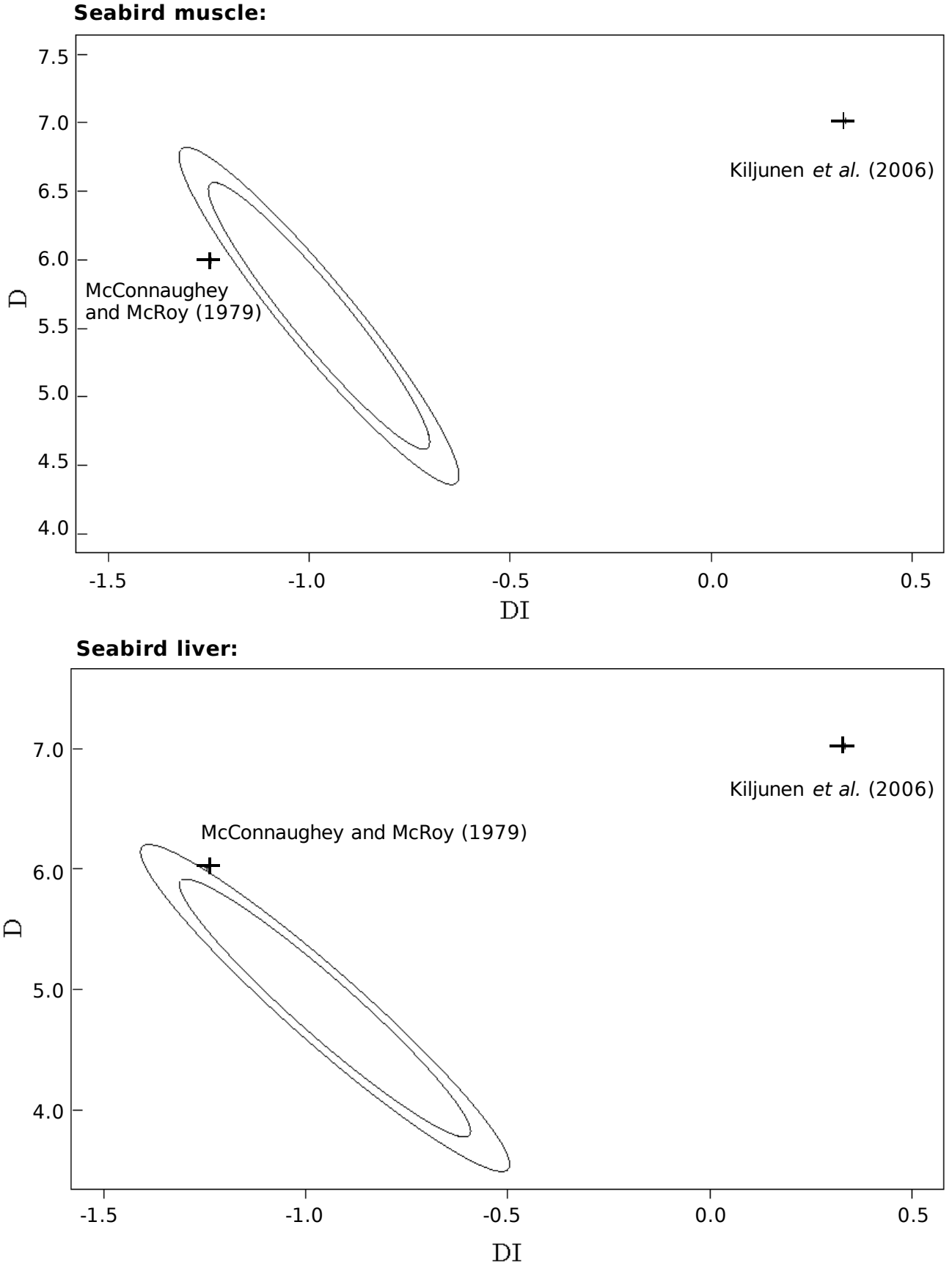

Seabird muscle:
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
D
McConnaughey
and McRoy (1979)
Kiljunen *et al.* (2006)
-1.5
-1.0
-0.5
0.0
0.5
DI
Seabird liver:
7.0
6.0
5.0
4.0
D
McConnaughey and McRoy (1979)
Kiljunen *et al.* (2006)
-1.5
-1.0
-0.5
0.0
0.5
DI

FIGURE 2b:

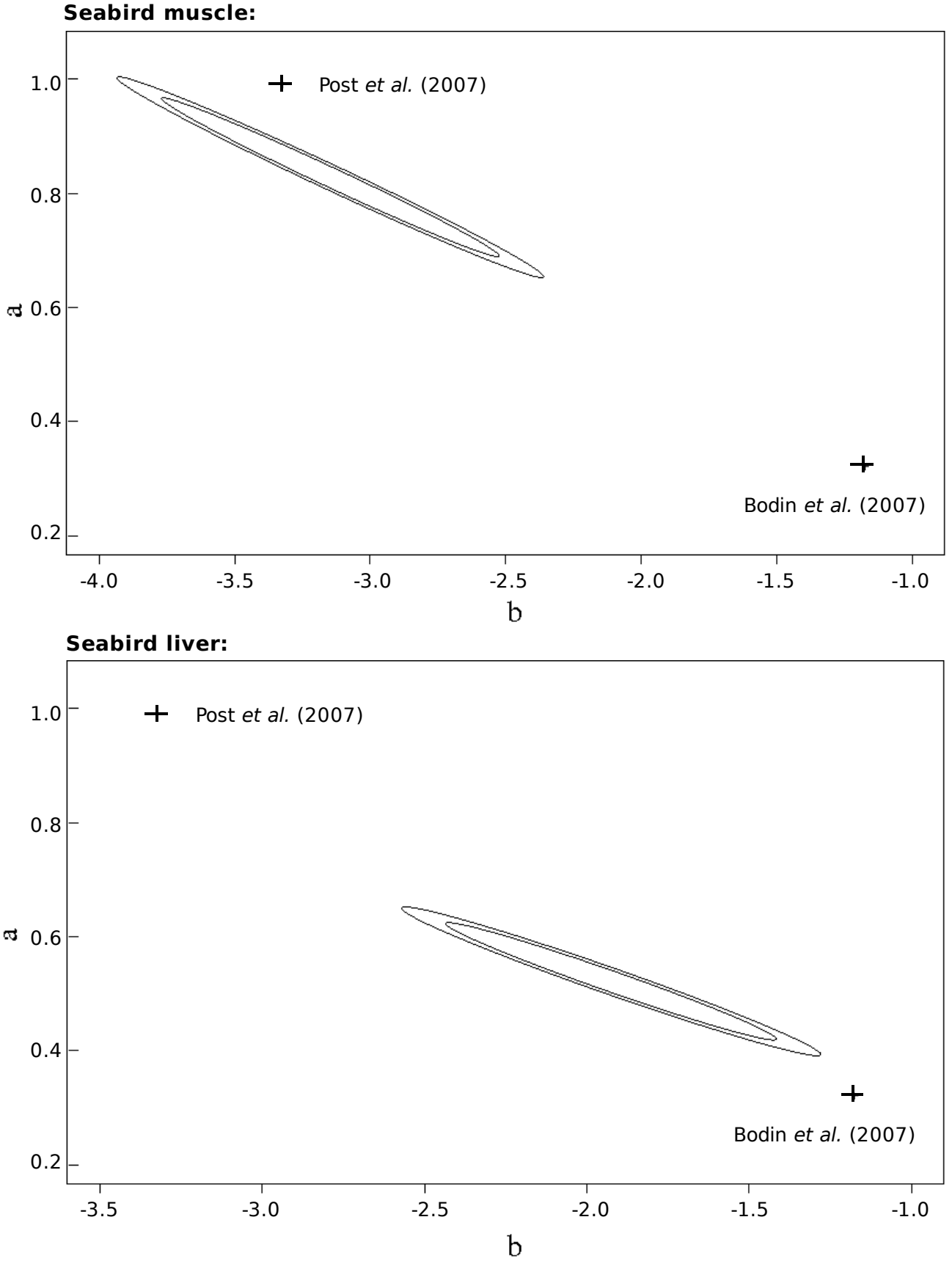
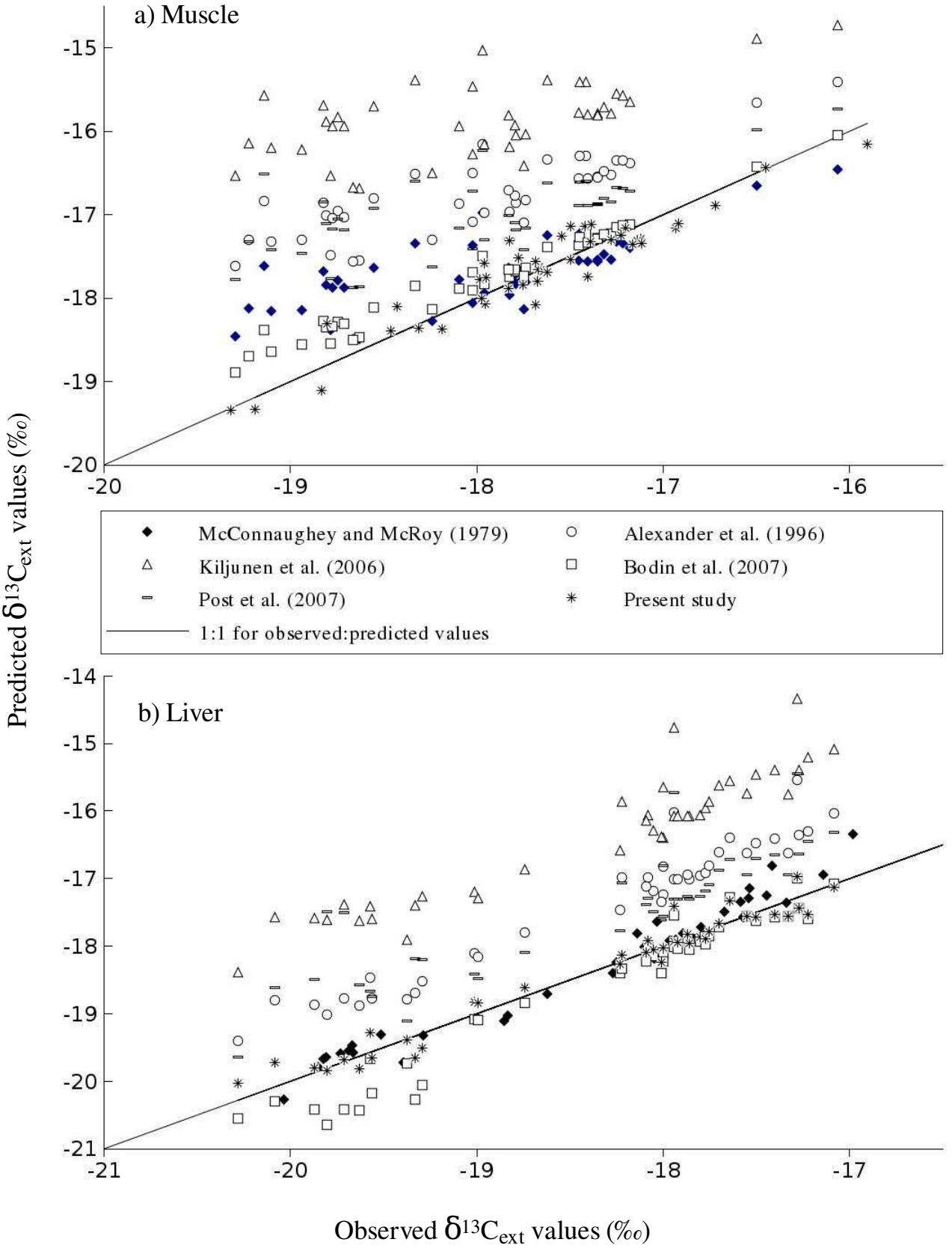

Seabird muscle:
Post et al. (2007)
Bodin et al. (2007)
a
b
1.0
0.8
0.6
0.4
0.2
-4.0
-3.5
-3.0
-2.5
-2.0
-1.5
-1.0
Seabird liver:
Post et al. (2007)
Bodin et al. (2007)
a
b
1.0
0.8
0.6
0.4
0.2
-3.5
-3.0
-2.5
-2.0
-1.5
-1.0

FIGURE 3:


a) Muscle
b) Liver
McConnaughey and McRoy (1979)
Alexander et al. (1996)
Kiljunen et al. (2006)
Bodin et al. (2007)
Post et al. (2007)
Present study
1:1 for observed:predicted values
Predicted δ13Cext values (‰)
Observed δ13Cext values (‰)